Regulation of hepatobiliary transport function by nuclear receptors

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Chapter 4

The human multidrug resistance 3 (*MDR3*) transporter is a peroxisome proliferator-activated receptor α (PPARα) target gene

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ABSTRACT

The human multidrug resistance 3 (*MDR3/ABCB4*) gene encodes an ABC transporter which is essential for phospholipid secretion into bile. Mutations in the *MDR3* gene cause progressive familial intrahepatic cholestasis (PFIC) type 3 and are associated with cholesterol gallstone formation. Peroxisome proliferator-activated receptor *alpha* (PPARα) is a ligand-activated transcription factor that belongs to the nuclear receptor family. Synthetic ligands of this receptor, i.e., hypolipidemic fibrates, induce the expression of the murine *Mdr2* (*Abcb4*) gene, which is the homologue of the human *MDR3*. Our aim was to characterize the human *MDR3* gene promoter with respect to potential regulatory actions of PPARα. Treatment of cultured human hepatocytes with various PPARα activators resulted in increased *MDR3* expression. The 5'-upstream region of human *MDR3* gene was examined for the presence of potential PPAR response elements (PPREs). A binding site for PPARα, arranged as a direct hexanucleotide repeat (DR1 motif), was localized ~4.9 kb upstream of the major transcription initiation site. This DR1 element was shown, by electrophoretic mobility shift assays, to bind the PPARα/9-cis-retinoic acid receptor (RXR) heterodimer, and mutagenesis of the DR1 motif abolished PPARα/RXR binding. Cloning of 2 copies of the PPRE upstream of a luciferase reporter gene increased luciferase activity by stimulation with PPARα, RXR and WY14,643. We have cloned ~5.5 kb upstream of the transcription initiation site, which will elucidate in ongoing transfection studies whether or not the human ABC transporter *MDR3* is a *bona fide* PPARα target gene.

INTRODUCTION

The *MDR3* (*ABCB4*) gene product, MDR3 P-glycoprotein (Pgp) plays an important role in the formation of bile. MDR3 Pgp is a phospholipid translocator[1], that belongs to the superfamily of ATP-binding cassette (ABC) transporters. MDR3 Pgp is predominantly expressed in the canalicular membrane of hepatocytes[2]. Through the actions of a number of ABC transporters, bile components such as bile salts, phospholipids and cholesterol, are secreted into the bile canaliculus. The secretion of phospholipids is of crucial importance for the protection of cellular membranes of the biliary tree against the high concentrations of detergent bile salts[3]. The *MDR3* gene shares homology with *MDR1* (*ABCB1*) and *BSEP* (*ABCB11*), and all three belong to the so-called B class of ABC transporters. The function of MDR3 has been elucidated by disruption of its homologue, i.e., the *Mdr2* gene, in the mouse. *Mdr2* knockout mice do not secrete phospholipids into bile and develop severe liver disease[1]. Progressive familial intrahepatic cholestasis (PFIC) type 3 in humans is due to mutations in the *MDR3* gene[4,5]. This is an inherited disease of childhood characterized by chronic cholestasis and jaundice, leading to cirrhosis. In addition, recent studies demonstrate that hetero- and homozygous *MDR3* gene mutations are also associated with intrahepatic cholesterol gallstone formation[6,7]. The human *MDR3* gene is localized on chromosome 7q21[8] and the promoter region of the *MDR3* gene has been characterized by Smit *et al.*[9].

Several lines of evidence indicate that murine *Mdr2* gene expression is tightly regulated at the transcriptional level by the peroxisome proliferator-activated receptor *alpha* (PPARα; NR1C1). PPARs belong to the family of nuclear receptors that are ligand-activated transcriptional regulators of gene expression. Fatty acids and their derivatives are natural ligands for PPARα[10]. Activated PPARα heterodimerizes with the 9-cis-retinoic acid receptor (RXR; NR2B1) and alters transcription of target genes after binding to specific peroxisome proliferator-response elements (PPREs). PPARα is highly expressed in the liver and controls the expression of genes involved in the β-oxidation of fatty acids and those encoding apolipoproteins A-I, A-II and C-III[11,12]. Previous studies by our group showed that upon treatment with ciprofibrate[13], which is a synthetic ligand for PPARα, *Mdr2* expression is increased at the mRNA, protein and functional level in wild-type mice, but not in PPARα-deficient mice. Furthermore, fasting for 24 hours[14], a physiological condition in which PPARα becomes activated, increased *Mdr2* expression in wild-type but not in PPARα-deficient mice. These results strongly indicate that PPARα is involved in the transcriptional regulation of murine *Mdr2.* Fibrates are widely used for treatment of hyperlipidemia in humans: the drugs lower plasma triglycerides and increase HDL cholesterol[11,15]. Fibrate treatment in humans may adversely affect bile composition, as they have been shown to affect expression of rate-limiting enzymes involved in bile salt synthesis[16-18]. The questions raises whether fibrate treatment in humans modulates *MDR3* gene expression. Therefore, we examined the 5'-upstream region of human *MDR3* gene for the presence of potential PPREs and investigated whether human *MDR3* expression is regulated by PPARα agonists. Our results indicate that *MDR3* is a positively regulated PPARα gene. We propose that this regulation occurs at the transcriptional level by binding of PPARα/RXR to a DR1 response element located at -4926 to -4913 in the promoter region of the *MDR3* gene.

MATERIALS AND METHODS

Steady-state mRNA levels in human hepatocytes determined by real-time PCR
Human primary hepatocytes were isolated as described previously[19] and incubated for the indicated times in dexamethasone-free Williams' E medium containing different PPARα ligands. Total RNA was isolated using TRIzol Reagent (Gibco BRL, Grand Island, NY) according to the manufacturer's instructions. Reverse transcription was performed on 2.5 μg of total RNA using random primers in a final volume of 38 μL (Reverse Transcription System, Promega, Madison, WI) for 10 min at 25°C, followed by one hour at 45°C. Samples were subsequently heated for 5 min at 95°C to terminate the reverse transcription reaction. Real-time quantitative PCR was performed on cDNA samples as described by Heid *et al*.[20] to quantify mRNA levels. The following primer and probe sequences were used for *ribosomal 18S*; sense primer 5'-CGG CTA CCA CAT CCA AGG A-3', anti-sense primer 5'-CCA ATT ACA GGG CCT CGA AA-3' and probe 5'-CGC GCA AAT TAC CCA CTC CCG A-3' (Accession number X03205), for *MDR3* (*ABCB4*); sense primer 5'-GAA GCA GAG GAT CGC CAT TG-3', anti-sense primer 5'-GTG GCC TCA TCC AGC AGA AG-3' and probe 5'-TGC CCT GGT TCG CAA CCC CA-3' (Accession number NM_000443), and for mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCoAS); sense primer 5'-CCA ATA GTG GAT GGG AAG CTT TC-3', anti-sense primer 5'-GCT TGC TTC CAC TGA TTC TGG A-3' and probe 5'-CCA AGG CCC GCA GGT AGC ACT G-3' (Accession number NM_005518). Primers and detection probes for the gene of interest, labeled with a fluorescent reporter dye (6-carboxy-fluorescein) and a fluorescent quenching dye (6-carboxy-tetramethyl-rhodamine), were added. Fluorescence was measured by an ABI Prism 7700 Sequence Detector v. 1.6 software (Perkin-Elmer Corp., Foster City, California). For every PCR reaction, *ribosomal 18S* was used as the internal control. The cycle number at the threshold (CT), after which the intensity of reporter fluorescent emission increases, was used to quantitate the PCR product.

Plasmid construction
hPPREx2-TKpGL3 was created by annealing the double-stranded oligonucleotide (5'-CCC GGG AGG TGC AGGGCAAAGGTCA GAT TCT AGG TGC AGGGCAAAGGTCA GAT TCT GCT AGC-3'), containing two copies of the PPRE (underlined nucleotides) found in the human *MDR3* gene promoter, by ligation into a SmaI-digested TK-pGL3 reporter vector. The construct was verified by restriction enzyme digestion and sequence analysis.

The reporter gene plasmid containing 5.5 kb of the MDR3 upstream regulatory region was constructed by using the clone CTD-3147L21 (Research Genetics; accession number AC079591), which contains the 5' part of the MDR3 locus. The first part of the promoter region, was amplified by PCR out of the CTD-3147L21 clone using the primers 5'-GC TCC AGC CTG ATC TCG GTC TT-3' and 5'-GAT CCA TGG CAG CCT GAG GAG AAA CC-3', which introduced a NcoI site at the ATG. The PCR amplification was performed using expand high fidelity enzyme (Gibco BRL) and a DMSO concentration of 8%. The resulting product was cloned into a pCR-Blunt II-TOPO vector (Invitrogen, Breda, the Netherlands). The second part of the promoter region, was amplified by PCR out of the CTD-3147L21 clone using the primers 5'-ACT CTC ACG GCT CTT ATG TGC TAC T-3' and 5'-AAC ACG TTT GCT CCA AGG TCA GAA C-3'. The resulting product was cloned into a pCR-XL-TOPO vector (Invitrogen). Subsequently, a NcoI/NarI fragment of

the first part and a KpnI/NarI fragment of the second part were, at the same time, cloned into a pGL3 basic plasmid (Promega, Madison, WY) digested with KpnI/NcoI. This resulted in a pGL3 basic containing 5.5 kb of the MDR3 5' upstream region. The construct was verified by restriction enzyme digestion and sequence analysis.

Cell culture and transient transfection assays

The human hepatoma cell line HepG2 was cultured at 37ºC in a humidified atmosphere of 5% CO_2 in air in Dulbecco's modified Eagle medium with GlutaMAX-1, 4.5 mg/L D-glucose, sodium pyruvate, pyridoxine supplemented with 10% (v/v) heat-inactivated fetal calfs serum, 100U/ml penicillin and 100 μg/mL streptomycin. HepG2 cells were transfected by the calcium phosphate coprecipitation method[21]. Plasmid DNA was isolated and purified using the Endofree Plasmid Maxi Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. For all assays, 3 x 10^5 HepG2 cells were transfected with 1 μg of the indicated luciferase reporter plasmid, 300 ng of pCMV-β-galactosidase expression vector, with or without 300 ng of the pSG5-hRXRα or pSG5-hPPARα expression vectors, as indicated. All samples were complemented with pGEM-5 (Promega, Madison, WY) to and identical amount of 4 μg/well. After transfection, medium was replaced with fresh medium containing WY14,643 or ciprofibrate and incubated for 24 hours. Luciferase activity was quantified using the luciferase assay system (Promega, Madison, WI) in a Microplate Luminometer (Berthold Detection Systems). β-Galactosidase activity was quantified with an enzyme assay system (Promega).

Electrophoretic mobility shift assays (EMSAs)

Human PPARα and human RXRα proteins were synthetized *in vitro* using the TNT Quick Coupled Transcription/Translation System (Promega, Madison, WI). Double-stranded oligonucleotides were end-labeled with [γ-^{32}P]ATP (Amersham, Little Chalfont, UK) using T4-polynucleotide kinase (New England Biolabs, Beverly, USA). The *in vitro* produced proteins, PPARα and/or RXRα, were incubated for 15 min at room temperature containing binding buffer (10 mM Hepes NaOH, pH 7.8; 100 mM NaCl; 0.1 mM EDTA; 10% glycerol; 1mg/ml BSA; 0.5 mM DDT), 1 μg of poly(dI-dC) and 1 μg of herring sperm DNA. The radiolabeled probes (DR1wt (5'-GAG CTG GAG GTG CAGGGCAAAGGTCA GAT TCT-3'), DR1mut (5'-GAG CTG GAG GTG CAGGGCAAA**CC**TCA GAT TCT-3'), where underlined nucleotides represent response elements half-sites and bases in boldface type are mutated) were added, and the binding reaction was incubated for a further 15 min at room temperature. The protein complexes were resolved by 4% nondenaturating polyacrylamide gel electrophoresis in 0.25x Tris-borate-EDTA (TBE) at room temperature. For competition experiments, the indicated excess quantities of unlabeled oligonucleotides were added to the binding reaction just before the labeled probes. The gel was dried and analyzed by autoradiography and PhosphorImaging.

RESULTS

PPARα activators induce *MDR3* expression in cultured primary human hepatocytes

To determine whether *MDR3* expression is regulated by PPARα, primary human hepatocytes were treated with different fibrates. An increase in *MDR3* mRNA expression was observed after treatment of cells with ciprofibrate (100 μM), bezafibrate (100 μM) and fenofibrate (100 μM) for 24 hours, as determined by real-time PCR (Figure 1A).

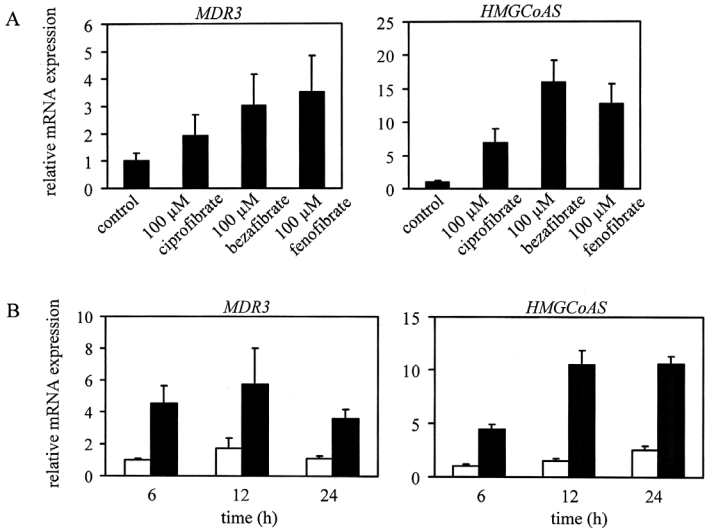

Figure 1. *Steady-state mRNA levels of MDR3 and HMGCoAS after fibrate treatment in human hepatocytes.* (A) Primary human hepatocytes were treated with 100 µM ciprofibrate, 100 µM bezafibrate, 100 µM fenofibrate or vehicle (DMSO) for 24 h. RNA was transcribed into cDNA and subjected to real-time PCR analysis as described in Materials and methods. (B) Primary human hepatocytes were treated with vehicle (DMSO) or 250 µM fenofibrate for 6, 12 or 24h. Values represent means ± SD.

Treatment with bezafibrate (250 µM) for 6, 12 and 24 hours also increased *MDR3* expression (Figure 1B) with maximal induction already after 6 hours. This increase was not seen in the vehicle-treated cells. As a positive PPARα-response gene, mitochondrial *HMGCoAS* (3-hydroxy-3-methylglutaryl-CoA synthase) involved in ketogenesis was used. Expression of this gene was also found to be induced upon fibrate treatment (Figure 1A and 1B).

PPARα activates the putative PPRE present in the *MDR3* gene promoter

The DNA sequences recognized by PPARα/RXR heterodimers usually comprise of a directed repeat separated by a single nucleotide (DR1). A database search identified a highly conserved DR1 located at -4926 to -4913 in the promoter region of the *MDR3* gene. This DR1 site was synthetized and cloned in two copies upstream of the luciferase reporter gene driven by the heterologous thymidine kinase promoter (TKpGL3) (Figure 2A) and cotransfection experiments were performed in HepG2 cells. Luciferase activity was increased upon cotransfection of the plasmid containing two copies of the DR1 with the human PPARα and RXRα plasmids and stimulated with WY14,643 (Figure 2B). These data indicate that the DR1 site in the *MDR3* promoter is an element by which ligand-activated PPARα may induce human *MDR3* promoter activity.

PPARα binds the PPRE within the *MDR3* gene promoter

To determine whether the PPARα/RXR heterodimer actually binds to the putative PPRE identified in the *MDR3* promoter, EMSAs were performed using the response element as a radiolabeled probe and the presence of *in vitro* translated human PPARα and/or human RXRα proteins. As expected, in the absence of PPARα and RXR, the probe did not bind

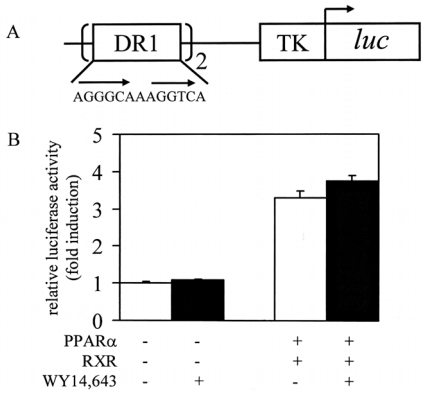

Figure 2. *Luciferase activity of the DR1 -4926 to -4913 response element by PPARα.* (A) Two copies of the wild-type PPREs were cloned upstream of the thymidine kinase minimal promoterdriven luciferase reporter (TKpGL3). (B) HepG2 cells were transfected with the hPPREx2-TKpGL3 plasmid, pCMV-β-galactosidase, and in the absence of presence of pSG5-hRXRα and pSG5-hPPARα. Cells were subsequently treated or not with WY14,643 (20 μM) for 24h. Values represent means ± SD.

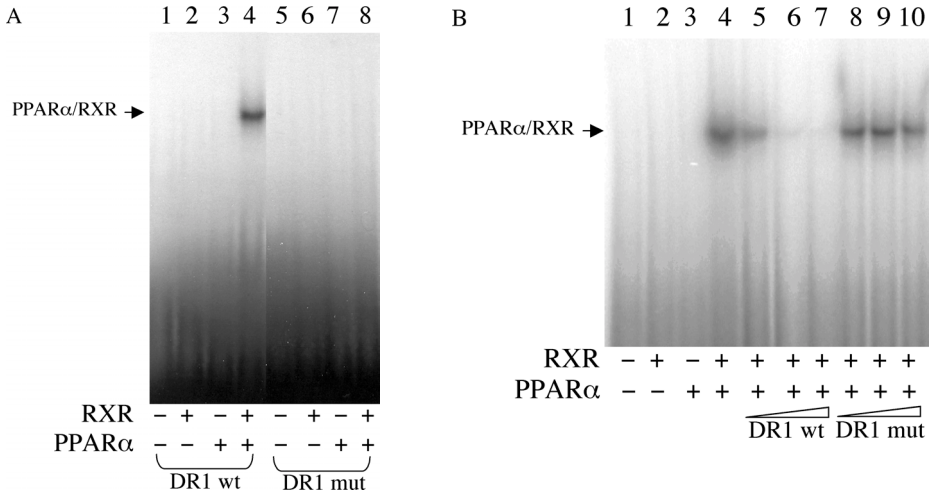

Figure 3. *Electromobility shift assay of binding of PPARα to PPRE.* (A) EMSAs were performed with end-labeled wild-type (wt) or mutant (mut) DR1 probes in the presence or absence of RXR and PPARα, as indicated. (B) Competition EMSA on radiolabeled DR1 probe was performed by adding a 1-, 10- or 100-fold molar excess of the indicated cold consensus DR1 or the mutant DR1 together with RXR and PPARα.

(Figure 3A and B, lane 2 and 3). In the presence of both PPARα and RXR the DR1 site was bound (Figure 3A and B, lane 4). No protein-DNA complexes were observed using the mutated DR1 (Figure 3A, lane 8). For competition experiments, increasing amounts (1-, 10-, and 100-fold excess) of either unlabeled wild-type DR1 or mutant DR1 were added to the binding reactions containing PPARα and RXR. PPARα binding to the wild-type DR1 was strongly competed by the wild-type DR1 itself (Figure 3B, lane 5-7). By contrast, the mutant DR1 oligonucleotides did not compete for PPARα binding to the DR1 (Figure 3B, lane 8-10). Taken together, these data demonstrate the PPARα binds as a heterodimer with RXR to the MDR3 PPRE site at positions -4926 to –4913. The next step of this study will be cotransfection studies with the cloned 5.5 kb promoter fragment of the human MDR3 promoter, which will elucidate whether the human MDR3 promoter is a *bona fide* PPARα target gene or not.

DISCUSSION

This study shows that the human *MDR3* gene is responsive to fibrates and possibly regulated by PPARα. Human hepatocytes treated with different PPARα agonists showed clearly increased *MDR3* expression. A nuclear receptor binding site for PPARα with the sequence 'AGGGCAAAGGTCA' was localized at ~4.9 kb upstream of the transcription initiation site. This DR1 element was able to bind PPARα/RXR, as was demonstrated by EMSA. Cloning of 2 copies of this DR1 element increased luciferase activity upon stimulation with PPARα, RXR and WY14,643. Stimulation with WY14,643, PPAR and RXR did not further increase luciferase activity compared to stimulation with PPARα and RXR alone. This is probably due to the presence of relatively high concentrations of endogenous ligands in the medium of the cells. A physiological condition in which PPARα becomes activated is during fasting and a functional reason for the induction of *MDR3* expression by PPARα could be a protective role of phospholipids against cytotoxic actions of bile salts which build up in bile ducts and gallbladder during fasting. Increased phospholipid secretion is able to protect the cellular membranes of the biliary tree against the high concentrations of detergent bile salts[3].

Until now, very little is known about the regulation of the human *MDR*3 gene. In a study by Jonkers *et al.*[22], hypertriglyceridaemic patients were treated with bezafibrate. They measured biliary lipid composition and showed increased biliary cholesterol concentration after bezafibrate treatment. This increase was observed together with a tendency to an increased percentage of biliary phospholipids as well as an increased molar ratio of phospholipids to bile salts. This phenomenon may have been caused by fibrate-induced *MDR3* gene expression, which functionally underscores the significance of our results.

The expression of the *MDR3/Mdr2* gene is tightly coupled to its function. *Mdr2* knockout (*Mdr2* $^{(-/-)}$) mice do not secrete phospholipids into bile and heterozygous mice (*Mdr2* $^{(+/-)}$) show a 40% decreased biliary phospholipid output[1]. In PFIC type 3 patients, in which functional MDR3 is absent, phospholipids are not secreted into bile[4,5]. In different forms of cholestasis, like cholestatic alcoholic hepatitis, MDR3 protein expression is not changed compared to healthy subjects, as analyzed by histology or immunofluorescence[23]. Patients with early stages of primary biliary cirrhosis (PBC) do not show changes in MDR3 expression, while patients with advanced stages of PBC have increased MDR3 expression levels[23,24]. In total parental nutrition (TPN)-induced cholestasis in adults, reduced biliary phospholipid excretion has been noticed, although no information on *MDR3* expression data is available so far[25]. In rodents, *Mdr2* expression appears to be unaltered under most conditions of cellular stress, e.g. it is not affected after endotoxin treatment[26] and only slightly enhanced after partial hepatectomy[27]. As shown in this and previous studies[13,28], the *MDR3/Mdr2* gene is responsive to fibrates. Besides fibrates, Mdr2 expression in rats is known to be increased upon treatment with statins (3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors)[29,30]. This could imply that sterol regulatory element-binding proteins (SREBPs) are involved in the regulation of rodent *Mdr2*, but this issue has not been solved yet. Bile salts, known to activate the bile salt-activated farnesoid X receptor (FXR), are also able to increase *Mdr2* expression[31,32]. A direct role of FXR in the regulation of the *Mdr2* gene is not likely, since *Mdr2* expression is also increased in FXR-deficient mice fed a cholate-enriched diet[32]. Nevertheless Huang *et al.*[33] showed very recently that an FXR response element in the human *MDR3* promoter is able to activate

the transcription of the human *MDR3* gene, as was demonstrated by luciferase studies. In contrast to our study, these authors made use of a transcription initiation site 5 kb upstream of the start site that we used. The study by Smit *et al.*[9] indeed has identified multiple transcription start sites for *MDR3*, but 'our' site was indicated to be the major transcription start site as analyzed by primer extension analysis. The upstream promoter, used by Huang *et al.*[33], was demonstrated to have no significant transcription in liver[9]. Besides a direct effect of FXR on transcription of the *MDR3* gene, the induction of *MDR3* expression by bile salts in human hepatocytes may also be explained by an induction of human PPARα by FXR: recently was shown that bile salts are able to stimulate PPARα expression via an FXR response element located within the human PPARα promoter[34].

Brown *et al.*[35] has identified that the transcription factor SP1 functionally interacts with the promoter of the *Mdr2* gene and it seems to be necessary for basal expression in rodents. We have shown[13] that ciprofibrate induced *Mdr2* expression in a PPARα-dependent manner in mice. This study shows that human *MDR3* gene expression is, at least partly, under control of PPARα. We propose that this regulation occurs at the transcriptional level by binding of PPARα/RXR to a DR1 response element located at -4926 to -4913 in the promoter region of the *MDR3* gene. Transfection studies of the cloned 5.5 kb promoter fragment of the human MDR3 will elucidate whether the DR1 motif in the human MDR3 promoter is responsive to PPARα or not. This is currently under investigation in our laboratory.

ACKNOWLEDGMENTS

We thank Olivier Barbier for his assistance. This work was supported by grant 902-23-191 from The Netherlands Organization for Scientific Research (NWO).

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